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Peripheral Circadian Oscillators

Interesting Mechanisms and Powerful Tools

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The lives of plants, animals, and human beings are all regulated by circadian clocks. In mammals, 24-hour rhythms of physiology and behavior are directed by a master clock in the suprachiasmatic nucleus (SCN) of the brain hypothalamus, which in turn entrains "slave" oscillators of similar molecular composition in most cells of the body. These peripheral clocks are interesting not only because they control many aspects of circadian physiology, but also because they are model systems through which we understand how the SCN regulates complex behavior. To this end, peripheral oscillators have been exploited both biochemically to understand the proteins that make up biological clocks, and genetically to decipher the ways in which individual differences in human chronotype might arise.

Key words: **circadian; clock; peripheral; chronotype; genetics**

Introduction

Rhythm, by definition, is change that is repeated in a similar pattern. In the environment, these changes occur in temperature, light/dark cycles, tidal rhythms, and in the seasons. Organisms have evolved mechanisms for anticipating these changes to maximize their survival and improve fitness. Thus, life moves in synchrony to the beat of clocks and calendars, some outside the body and some within the very cells of all living things. Circadian clocks are those that have an intrinsic period length of approximately 24 hours (from the Latin *circa diem*, "about a day").

The earliest circadian clocks probably evolved in Kingdom *Archaea*. At least, modern diazotrophic cyanobacteria display daily rhythms of nitrogen fixation in light/dark cycles and in constant darkness.[1–3] These oscillations have been shown to be important to adaptive fitness in normal light/dark conditions, probably because of the chemical incompatibility of photosynthesis and nitrogen-fixation pathways.[4] While the adaptive benefit of circadian clocks to more complex eukaryotes is less clearly defined and tested, their presence and conservation in most branches of Domain *Eukarya* is clear. Thus, the molecular genetic bases of circadian rhythms have been investigated extensively in many model organisms.

In mammals, the circadian oscillations of gene expression that are orchestrated by these clocks influence nearly all aspects of physiology and behavior, including sleep/wake cycles, the cardiovascular system, body temperature, endocrinology, renal and hepatic function, and the activity of the digestive tract. In total, 10% of all genes are expressed in circadian fashion.[5–7] Recent studies suggest that posttranscriptional regulation may increase this fraction even further, as high as 20% in some tissues, such as liver.[8]

Concepts of Molecular Clockwork

Core clock components are defined as genes whose protein products are essential for the generation of circadian rhythms.[9] The process of understanding the architecture of these components as an oscillating clock began with the discovery of clock mutants in the filamentous fungus *Neurospora crassa* and the fruitfly *Drosophila psuedobscura*. The identification of *Neurospora* clock mutants[10] led to the discovery and cloning of the Frequency locus (*frq*), one of the first known circadian clock-genes.[11] Similarly, in *Drosophila*, the pioneering forward genetic screens of Roland Konopka and Seymour Benzer led to the discovery of the Period (*per*) locus.[12,13]

Subsequent research in both organisms showed that their circadian oscillators are based upon autoregulatory feedback loops of transcription and translation.[14] This basic structure has been conserved in all species studied. Interestingly, however, these loops are not essential in cyanobacterial clocks, which are based upon

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cyclical rhythms of phosphorylation of clock components.[15] Although cyclical posttranslational modifications of clock components play a prominent role in metazoan clocks as well, current evidence suggests that transcription/translation feedback loops consisting of both positive and negative elements are essential to their primary mechanism.[16]

In mammals, the positive elements of these loops include members of helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) transcription-factor family, CLOCK and BMAL1. In some tissues including brain and liver, the NPAS2 protein can also play an important role.[17,18] CLOCK or NPAS2 and BMAL1 heterodimerize and initiate transcription of target genes that contain E-box *cis*- regulatory sequences. Negative feedback is achieved by a complex of other components including the PERIOD1–3 (PER) and CRYPTOCHROME1–2 (CRY) protein products. The genes encoding these products are activated by CLOCK:BMAL1 heterodimers via E-boxes, and their cognate proteins then translocate back to the nucleus to repress their own transcription by preventing CLOCK:BMAL1 complex binding.[14,19] The CIPC protein, which interacts with CLOCK:BMAL1 complexes and inhibits their activation activity, probably also plays a role.[20]

A second type of loop is formed by CLOCK:BMAL1 heterodimers that activate transcription of retinoic acid-related orphan nuclear-receptor genes *Rev-Erb*α and *ROR*α.[21] REV-ERBα and RORα subsequently compete dramatically to bind retinoic acid-related orphan receptor-response elements (RORE*s*), which are present in the *Bmal1* promoter. RORα (as well as related proteins RORβ and γ) activate transcription of *Bmal1*, and REV-ERBα (and probably its sister protein REV-ERBβ) repress it.[21,22]

Posttranslational modifications of clock components and of other proteins play an important role in both loops. These modifications include phosphorylation and ubiquitiniation of clock components, chromatin modifications, and possibly even direct acetylation of some clock components by others. For example, PER proteins are phosphorylated by casein kinase 1 ε and δ, and probably by other kinases as well, and these phosphorylations affect both nuclear localization and degradation via ubiquitination.[23] Ubiquitin ligase coupling via the FBXL3 protein also affects degradation of other clock proteins such as CRYs.[24,25] At the level of chromatin structure, circadian loci such as *Dbp* and *Rev-Erb*α change each day from a repressive to an active chromatin structure via histone acetylation and methylation.[26] Finally, the CLOCK protein itself possesses a histone acetyltransferase activity and can acetylate BMAL1.[27] Although it has been speculated that posttranslational modifications might themselves suffice for circadian oscillations in metazoans (as in the cyanobacterial clock, which can operate independent of transcription), no experimental evidence exists so far to support this idea.

Central Clocks and Peripheral Oscillators

Both conceptually and physically, biological clocks can be divided into three main parts: an input pathway that relays signals from the external environment to the clock; a central oscillator or pacemaker that is able to generate and sustain rhythms, as well as receive and integrate signals from input pathway; and an output pathway by which the oscillator can affect physiology. In the absence of external timing cues, the central oscillator continues to cycle with a "free-running" period of approximately 24 hours, and the many biological processes controlled by the output pathway remain rhythmic. Under normal conditions, however, the pacemaker is continuously adjusted to external 24-h light/dark cycles, the "photoperiod." Light is a very strong zeitgeber (timing cue), and light-induced phase shifts reset the pacemaker's oscillation. Advances or delays occur because the pacemaker is differentially sensitive to light exposure at different times of the free-running circadian cycle.

The master pacemaker in mammals is located in the suprachiasmatic nuclei (SCN), approximately 16,000 neurons located in the ventral part of the hypothalamus.[28,29] Electrophysiological studies have demonstrated that circadian oscillations in the SCN are generated in individual neurons in a cell-autonomous fashion.[30] Photic information is received by the cells of the retina and reaches the SCN via the optic nerve and the retinohypothalamic tract. Here, it adjusts the phase of the molecular clock in the SCN. This phase adjustment may involve the activation of the clock-genes *Per1* and *Per2* in immediate-early fashion—that is, without the need for prior protein synthesis—upon light stimulation.[31,32] The SCN then communicates this timing information to the rest of the body. The mechanism by which this signal relay occurs has been a subject of much recent debate.

Core clock-genes are expressed rhythmically not only in the SCN but also in most cells of the body. In fact, oscillators that are capable of generating at least several regular cycles of circadian gene expression were found in peripheral, nonneural tissues of multiple

animals, including *Drosophila*,[33] *Zebrafish*,[34] and mammals.[35] Furthermore, Balsalobre *et al*. found that brief treatment of immortalized fibroblasts with high concentration of serum, induces circadian gene expression that persists for several days.[36] Similar results were obtained when the cells were incubated with chemicals that can activate different signal transduction pathways [e.g., tissue plasminogen activator (TPA), which activates protein kinase C and MAP kinases;[37] forskolin, which activates protein kinase A; and dexamethasone, a glucocorticoid hormone analog[38,39]]. From these data, it was immediately imagined that the SCN clock can entrain the phase of peripheral clocks through chemical cues, and that these oscillators in turn control circadian physiology. In fact, the situation proved to be much more complicated, and much more interesting. In simpler animals such as *Drosophila* and zebrafish, nearly all organs of the body are independently light sensitive. Thus, dismembered organs not only continue to display circadian patterns of gene expression but also entrain independently to environmental light/dark cycles.[40] In these organisms, it is believed that the central clock tissue (in *Drosophila*, the lateral neurons) controls locomotor behavior,[41] but peripheral clocks independently control physiology of their respective organs and cells in synchrony with the environment.

By contrast, the mammalian circadian system is organized into a strict hierarchy of oscillators. The main oscillator is localized in the suprachiasmatic nuclei of hypothalamus. As mentioned previously, light is perceived in a strictly ocular fashion by both traditional rods and cones and nontraditional opsin photopigments in retinal ganglion cells,[42] and is transmitted to the SCN. From here, current research suggests that a redundant web of direct and indirect signals can transmit this signal to peripheral organs. At least the initial signal is probably hormonal, because Silver and colleagues showed that a transplanted suprachiasmatic nucleus encapsulated in porous material can rescue circadian locomotor rhythm in a lesioned acceptor hamster.[43] Nevertheless, this unexpected result proved to be only the beginning of a very interesting story to which many labs have recently contributed.

One way in which the SCN probably influences circadian physiology and gene expression is via the pituitary–adrenocortical axis, specifically via glucocorticoid hormones. Glucocorticoids have many important functions, including regulation of glucose, fat, and protein metabolism. They also have anti-inflammatory actions, and can affect mood and cognitive functions. Glucocorticoids can bind glucocorticoid receptor (GR), a nuclear hormone receptor found in many cell types but not in the SCN.[44] It has been shown that dexamethasone, a glucocorticoid analog, can induce *Per1* expression in RAT1 fibroblasts, as well as change the phase of circadian gene expression in peripheral tissues, but not SCN. It is clear that glucocorticoids are not the sole entraining signal from the SCN, because mice lacking GR in the liver possess normal circadian rhythmicity in this organ.[45]

Another dominant zeitgeber, or timing cue, for peripheral circadian clocks is food itself. It was shown that the expression profile of many circadian genes in the liver and other peripheral tissues is influenced by the timing of food intake. Specifically, restricted feeding uncouples peripheral circadian gene expression from that in the suprachiasmatic nucleus.[46,47] The speed and the degree to which an organ changes its circadian rhythm to match the timing of food uptake varies among different organs. Interestingly, however, this phase shift happens more quickly in adrenalectomized animals or in tissues lacking the glucocorticoid receptor when feeding time and photoperiod are placed in opposition.[48] Therefore, it is likely that the twin signals of feeding time and glucocorticoid secretion act separately *in vivo* to set clock phase. The exact nature of the food-induced signal is unclear, but the observation that glucose alone can phase shift circadian gene expression in cultured cells *in vitro* suggests that basic food metabolites could suffice.[49] Once again, it is unlikely that food entrainment is the only timing signal *in vivo*, because mice that are fed frequent isocaloric meals still display robust circadian rhythmicity in peripheral organs.[50]

A third basic class of signal that may entrain peripheral oscillators is fluctuation in body temperature. In heterotherms such as *Drosophila*[51] and *Neurospora*,[52] it has been known for some time that shallow 24-h temperature fluctuations—for example, 12 h at 37°C followed by 12 h at 33°C—can synchronize and phase-shift circadian oscillations in behavior and gene expression. More recently, however, we have shown that rhythmic body temperature can sustain peripheral circadian oscillators, and that inversion of temperature cycles in the liver or brain cortex can invert circadian gene expression in these organs without affecting the phase of the SCN.[53] In spite of these promising results, temperature is also not the sole source of peripheral circadian entrainment *in vivo*, because "scrambling" of temperature cycles does not result in a loss or dampening of peripheral circadian rhythmicity.

Finally, Okamura and colleagues showed recently that communication between SCN and peripheral tissues can occur via a fourth channel: the sympathetic nervous system. In their study, periodically injected

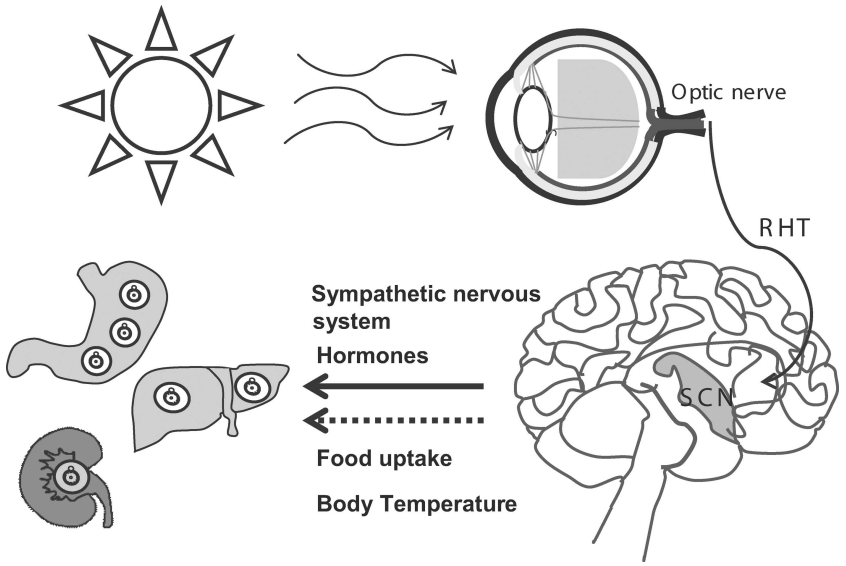

FIGURE 1. Organization of central and peripheral oscillators. Light is the principal timing cue that synchronizes the neurons located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, whereas the SCN communicates with peripheral oscillators via a redundant web of direct and indirect signals. For the circadian system, light is received by both rod and cone cells containing traditional opsin pigments and retinal ganglion cells containing melanopsin. This message is sent to the SCN via the optic nerve and the retinohypothalamic tract. From the SCN, a variety of signals are transmitted to peripheral oscillators. These include direct signals from the sympathetic nervous system, hormonal cues, and indirect signals such as food metabolites and body temperature that are relayed via the control of the SCN over appetite and locomotor activity.

adrenaline in SCN-lesioned mice induced strong *Per2* expression in the liver. This result suggested adrenergic regulation influences circadian clock-gene oscillations in peripheral tissues. Moreover, electrical stimulation of the sympathetic nerve increased *mPer1* transcription mainly in the liver of these mice. As a confirmation that the sympathetic nerve is important for sustaining *Per* gene expression in peripheral organs, the mice were injected by 6-hydroxydopamine-HCl, which causes the destruction of sympathetic nerves. The expression of the *Per2* gene was significantly reduced. Together, these studies demonstrated that sympathetic nerve activity plays an important role in the delivery of the central clock information to at least some peripheral tissues.[54]

Of course, although we have evoked four distinct channels of communication in the preceding discussion, significant overlap is possible and even likely. For example, corticosterone release from the adrenal gland can occur via the sympathetic nervous system, and food metabolites can be brain-signaling molecules. Overall, the important message is that the SCN conveys its timing information to peripheral circadian clocks by a redundant mix of direct hormonal and nervous signals, and indirect environmental ones, such as feeding and body temperature, that are influenced by rest/activity cycles (FIG. 1).

Circadian Physiology

A related but separate question is how the SCN directs circadian physiology and gene expression. cDNA microarray technologies suggest that the circadian clock regulates the transcription of about 10% of all genes expressed in each tissue.[5–7] Given the pervasive and cell-autonomous nature of peripheral circadian oscillators, it was largely assumed that they would be responsible for the direction of most circadian physiology and gene expression. Certainly, this hypothesis is at least partially true. Many clock-controlled genes are directed by the same *cis*-acting elements such as E-boxes that control expression of clock-genes themselves. For such "direct" regulation, the *Dbp* gene has served as an excellent model system.[55] Ripperger *et al*. have dissected this gene and shown that it possesses multiple E-boxes in both promoter and intronic regions. By clock proteins and chromatin modifying factors that bind to these regulatory elements, the entire locus is

switched from an active to an inactive chromatin conformation to turn the locus on and off in circadian fashion.[26] Meanwhile, DBP is itself a member of a family of circadian transcription factors that direct the expression of other circadian output genes important for the liver's role in xenobiotic detoxication.[56] Thus, one way in which the circadian clock controls circadian physiology is by transcription factor cascades directed by peripheral oscillators.

A second way in which the SCN drives circadian physiology is via direct nervous connections to other centers of the brain. The major output from the suprachiasmatic nuclei is the dorsomedial nucleus and supraventricular zone. The dorsal supraventricular zone can organize circadian regulation of body temperature. The ventral suprachiasmatic zone regulate circadian rhythms of sleep and wakefulness, and the dorsomedial nucleus is necessary for feeding activity.[57] Since an SCN encapsulated in porous plastic material can rescue behavioral arrhythmicity in lesioned animals,[43] multiple laboratories have also tried to purify neuropeptides important for SCN signaling. Kramer and Weitz identified one such factor as transforming growth factor α (TGF-α). This growth factor activates the epidermal growth-factor receptor (EGFR) on neurons in the hypothalamic supraventricular zone, and these may mediate circadian locomotor activity.[58]

Surprisingly, recent tissue-specific clock knockouts have shown that the SCN can also directly regulate some peripheral circadian physiology at the gene-expression level. The elegant experiments of Kornmann and colleagues showed that some circadian expression of genes in peripheral organs can be driven directly by systemic circadian signals and others by local oscillators. To show this, they generated mice with tetracycline-dependent hepatocyte clocks by placing the clock-gene *Rev-Erb*α under the control of the tetracycline operator. Its overexpression in the absence of doxycycline thereby leads to silencing of *Bmal* transcription, and with it circadian clock function in this tissue. When the hepatocyte clocks are turned off, the bulk of circadian transcription in the liver is strongly attenuated. This result indicates that most expression of circadian liver genes is driven by local cellular clocks. By contrast, a smaller subset of genes—which included the clock-gene *mPer2*—continued to oscillate. Hence, these genes must be regulated by systemic signals such as hormones, metabolites, or temperature.[59]

Circadian Behavior

The circadian system has a very important influence on human physiology and behavior. Indeed, considering the extent of circadian regulation described earlier, it is perhaps not surprising that disruption of biological clocks has a negative effect. One of the most obvious manifestations is jet lag, misadjustment of circadian phase due to travel. Links have also been established between circadian irregularities and psychiatric disorders, including various forms of depression and mania. Prolonged disruption of circadian rhythms is believed to have significant adverse health consequences on peripheral organs outside the brain as well, particularly in the development or exacerbation of cardiovascular disease and cancer.[60–62] Conversely, chronopharmacology—the timing of treatment in coordination with the body clock—may significantly increase efficacy of various therapies, and reduce drug toxicity or adverse reactions.[63]

Even under normal conditions, the complex nature of circadian behavior is evident from the fact that phasing of the cycle during the day varies widely for individuals, resulting in extremes colloquially called larks and owls. Morningness/eveningness, or "chronotype," is an individual characteristic that refers mostly to the phase of sleep timing.[64] Because of this effect of the circadian system upon sleep, most circadian rhythm disorders are therefore classified as sleep disorders. Nevertheless, circadian sleep disorders and true sleep disorders are likely to be mechanistically unrelated, and therefore it is both scientifically and clinically relevant to distinguish between them.

Sleep is an active and as yet poorly understood process, during which many physiological and cerebral events occur. Indeed, even sleep itself is actually an ultradian process represented by the alternation of different electrophysiologically defined sleep states. In general, the daily sleep/wake cycle is under circadian control, although the urge to sleep appears to be controlled by brain functions that are independent of the circadian system.[65] This independence led Borbely and colleagues to propose a model for the regulation of sleep that includes a homeostatic process that accumulates during wakefulness and diminishes during sleep, as well as an independent circadian drive.[66] Each of these processes can operate independently; thus, sleep duration is not correlated with sleep phase in humans.[67]

So-called "circadian rhythm sleep disorders" can result from alterations in the properties of the endogenous circadian clock (e.g., delayed sleep phase and advanced sleep phase) or changes in the physical environment in relation to the endogenous clock (shift work disorders and jet lag). In the former class, which is unrelated to human choice, genetic variations in circadian genes have been found to associate with these disorders.

TABLE 1. Genetic variations in circadian clock-genes are associated with behavioral disorders

Disease	Location of mutation or SNP	Affected gene
Familial advanced sleep-phase syndrome (FASPS)[70]	662 S/G	*Per2*
	44 T/A	*Ck1δ*
Extreme diurnal preference[115]	T2434C	*Per1*
Bipolar disorder (BD)[82,116]	11p15	*Bmal 1*
	1p36.23	*Per3*
	12q12-q13	*TIMELESS*
Delayed sleep-phase syndrome (DSPS)[73]	647 V/G	*Per3*
	S408N	*CK1ε*
Seasonal affective disorder (SAD)[117]	471 L/S	NPAS2
Non-Hodgkin's syndrome (NHS)[118]	394 A/T	*NPAS2*
Schizophrenia[119]	31111 T/C	*Clock*
Winter depression[120]	SNP 10870	*Per2*
	SNP rs2290035	*Arnt1*

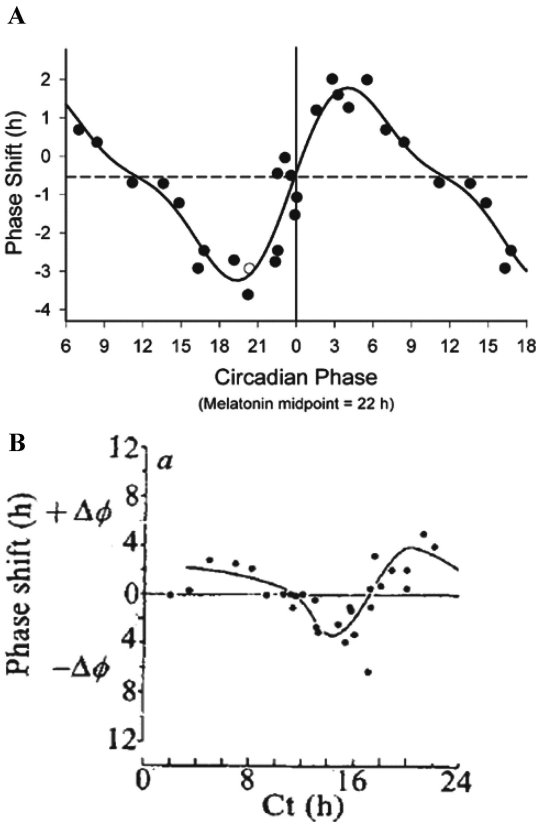

FIGURE 2. Phase-response curves in different organisms. (**A**) The phase-response curve to a bright light stimulus in humans. Circadian phase was measured as the midpoint of melatonin expression. Positive values reflect phase advances, and negative values reflect phase delays. (Panel reproduced by permission from Khalsa *et al*.[91]) (**B**) Phase-response curve to a bright-light stimulus in the cockroach *Nauphoeta cinerea*. Circadian phase was estimated by the timing of locomotor activity. (Panel reproduced by permission from Saunders and Thomson.[121])

TABLE 1 shows a list of polymorphisms that have been linked to known clock-genes. One of the most-studied syndromes is familial advanced sleep-phase syndrome (FASPS). Individuals with this syndrome can wake up and go to sleep hours earlier than normal. This phase change is believed to be related to a change in the endogenous free-running period of the human circadian oscillator. Normally around 24 hours, it has been measured to be only 20 hours in an individual from an extensively studied FASPS lineage.[68] In this lineage, the source of the circadian change has been mapped to a change from serine to glycine at residue 662 of the *Per2* gene. This mutation abolishes one of the phosporylation target sites for CK1ε. *In vitro* experiments confirm that the mutation reduces the ability of CK1ε to phosphorylate PER2 protein.[69] A second independent lineage confirmed the importance of casein kinase-mediated phosphorylation to human chronotype. This time, the corresponding mutation was mapped to a missense (T44A) change in the CK1δ locus that results in lower kinase activity *in vitro* and shorter periods when introduced into mice *in vivo*.[70]

Not surprisingly at all, not only extreme early phase but also extreme late phase has been correlated with genetic alterations in clock-genes. For example, a genetic association study of 105 individuals has linked a length polymorphism in the *Per3* gene to DSPS.[71] Other studies have seen various degrees of association, not only between DSPS and this *Per3* allele,[72] but also other *Per3* alleles[73] and a *CLOCK* allele,[74] though not all studies have drawn the same conclusions.[75] In addition, an allele of CKIε has been found to be anticorrelated with DSPS, implying that it may play a protective role against this syndrome. Although the basis of altered chronotype in this suite of mutations is unknown, many of them are presumed to alter endogenous period length in fashions similar to FASPS, principally because there is an association between free-running period length and entrained behavioral phase in humans and in other animals.[76] Moreover, genetic mapping studies in inbred strains of laboratory mice suggest that many different loci other than those of known clock-genes might influence free-running behavioral period.[77]

One striking feature of circadian rhythm sleep disorders is that they are often associated with other mood disorders. Indeed, a part of this association is by definition: an established clinical symptom of diseases like major depressive disorder (MDD) and BD is abnormal sleep/wake, appetite, and social rhythms,[78,79] which are also hallmarks of circadian rhythm disorders. Nevertheless, an increasing body of evidence suggests that there exists an interesting genetic basis for this correlation. In bipolar patients, a single nucleotide polymorphism in the 3′ flanking region of the *Clock-gene* associates with a higher recurrence rate of bipolar episodes.[80] This mutation is specific to bipolar depression: a similar association is not found in MDD (or unipolar depression).[81] Another mutation, this time linked to the onset of illness in BD, has been localized to the glycogen synthase kinase 3β promoter.[82] This enzyme is the target of lithium, a common treatment for BD, and can phosphorylate the clock component REV ERBα.[83]

It is likely that multiple other genetic associations remain to be found between the various forms of depression and clock-genes. A pilot study of circadian genes and their linkage to BD 1 unearthed *Bmal1, Timeless*, and *Period3* as possible candidates.[84] Schizophrenia is also accompanied by severe sleep/wake disturbances, and has been associated with clock-gene polymorphisms in this and other preliminary studies.[85] Finally, dementia has also been associated with circadian dysfunction in Huntington's disease (HD), though in this case the dysfunction is believed to be a neurological consequence of HD pathology upon the SCN, rather than a genetic linkage between dementia and circadian rhythm disorders.[86]

Measurement of Human Circadian Clocks

One of the principal difficulties in determining the genetic linkage between human behavioral disorders and the circadian oscillator is simply measuring the properties of the human circadian clocks that determine behavior. In principle, two different properties can be measured: free-running period, or the length of one oscillation under constant environmental conditions, and phase response/entrainment, the ability of the clock to alter its phase in response to external stimuli. Though formally distinct, these two properties are under normal circumstances inter-related because the mechanism by which circadian clocks are synchronized by light is non-parametric.[51] In other words, to entrain to the daily light/dark cycle, the circadian oscillator responds differently to light at different phases of its cycle. This differential effect is most easily visualized as a phase-response curve (PRC), which plots phase shifts of a circadian rhythm as a function of the circadian phase that a stimulus, or zietgeber, is given. The characteristic form of this curve was first described by DeCoursey 30 years ago in the flying squirrel,[87] and can be determined by a number of different protocols, as described by Aschoff.[88] From such a curve, one can make deductions about the phase, period, and amplitude of the central oscillator (FIG. 2).

In human beings, the measurement of either the free-running period or phase response is very expensive and labor-intensive because it demands extensive subject observation under controlled laboratory conditions. Nevertheless, reliable estimates have been made by a variety of methods for both human period length (24.2–24.4 hours)[89,90] and the human phase response to bright light pulses.[91] By comparing human free-running period length to behavioral chronotype, it has also been possible to observe a correlation between these properties.[76,92] Similar observations of morning-type behavior in individuals of short endogenous period and evening-type behavior in individuals of long endogenous period have been observed previously in other animal systems.[93]

PRCs can also be performed with other phase-shifting agents such as drugs or temperature. For example, a physiological dose of the hormone melatonin shifts circadian rhythms in humans according to a phase-response curve (PRC) that is nearly opposite in phase with the PRCs for light exposure: melatonin delays circadian rhythms when administered in the morning and advances them when administered in the afternoon or early evening. This difference points to multiple different pathways for the entrainment of the human circadian oscillator. More practically, the human melatonin PRC also provides critical information for using melatonin to treat circadian phase sleep and mood disorders.[94]

Peripheral Oscillators as Tools to Study Human Behavior

Although the central clock of the SCN that specifies behavior is quite difficult to access at a molecular level, the circadian clocks that exist in peripheral cells appear to use many of the same components. Hence, a major breakthrough for mammalian circadian biologists has been the ability to use these cells as proxies—albeit imperfect ones—for the clocks of the SCN. The period of electrical firing in the SCN has been observed to

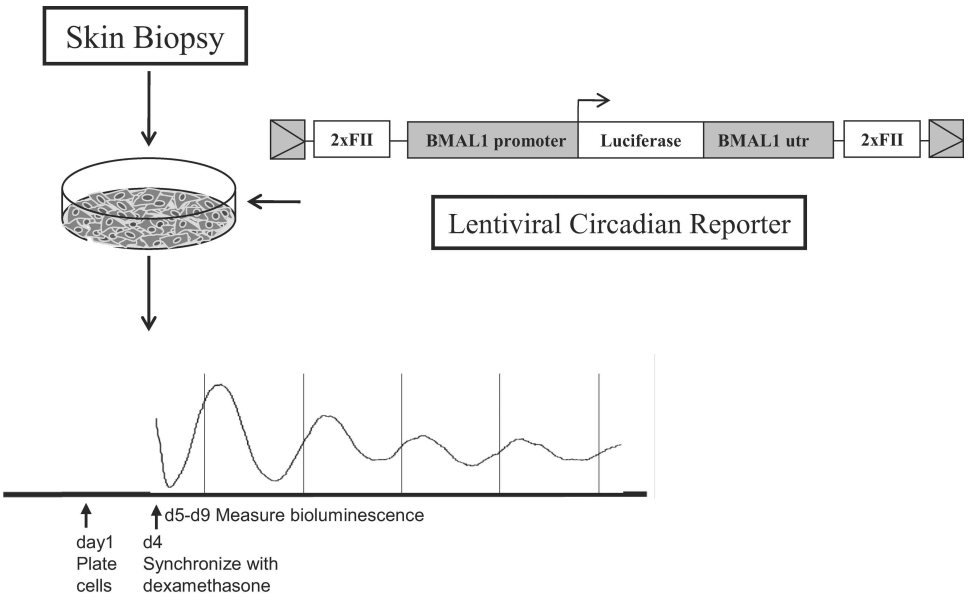

FIGURE 3. Measurement of circadian rhythms from skin fibroblasts. Dermal fibroblasts are cultivated from a 2-mm dermal punch biopsy, and then infected with a lentivirus harboring a luciferase reporter gene driven by a circadian promoter. Subsequently, circadian oscillations in a plate of infected cells are synchronized with a chemical signal such as dexamethasone, and the free-running circadian period is measured as the period of circadian bioluminescence using a photomultiplier measurement apparatus. (Figure adapted from Brown *et al*.[98])

correlate closely with the period of behavior in hamsters,[95] so it is possible that there exists a direct parallel between human circadian behavior and the molecular properties of human peripheral oscillators. Importantly, genetic differences appear to manifest themselves in both peripheral and central oscillators.[96,97] Our laboratory has compared the wheel-running behavior of strains of mice displaying different circadian periods with the circadian period of fibroblast gene expression in the same animals, and we have observed a correlation between the two.[98] (See TABLE 2.)

Although the clocks of peripheral and central oscillators are similar, they are not identical. In mice containing a PER2:luc fusion protein, the free-running period of luciferase expression varies by up to 3 hours in explants from various tissues. Fibroblast period was one of the closest to that of the SCN.[99] Nevertheless, genetic differences appear to be exaggerated in fibroblast a circadian period compared to that of the SCN. For example, the disruption of the *Per1* gene in mice results in a 1-h shortening of the behavioral circadian period in the mouse, but a 4-h reduction in the period of fibroblast gene expression.[96,98] Similar exaggerations can be seen for many other circadian mutations.[96,98] Recent research suggests that this increased robustness of the central clock versus peripheral clocks is due to intercellular coupling of neurons within the SCN. This coupling can occur via both neuropeptidergic mechanisms and electrical synapses.[100,101] Another source of difference between SCN and peripheral oscillators may occur because of their use of slightly different suites of circadian clock proteins. For example, the CLOCK protein appears to be essential to proper clock function in peripheral oscillators, but dispensable for SCN oscillations and circadian behavior *in vivo* in

TABLE 2. Comparison of the period of wheel-running behavior with that of mouse fibroblast period length in various mouse strains

Genotype	Wheel-running behavior (hours)	Period of fibroblast luminescence (hours)
Wild type BI 6	23.8 ± 0.1	24.4 ± 0.4
Per1$^{brdm/brdm}$	23.4 ± 0.1	20.0 ± 0.1
Per2$^{brdm/brdm}$	23.0 ± 0.6	Arrhythmic
Per1$^{brdm/brdm}$, Cry2$^{-/-}$	24.7 ± 0.2	25.9 ± 0.8
Cry2$^{+/-}$	23.6 ± 0.1	23.6 ± 0.21
Cry2$^{-/-}$	24.6 ± 0.1	25.4 ± 0.7
Per2$^{brdm/brdm}$, Cry1$^{-/-}$	Arrhythmic	Arrhythmic
Per2$^{brdm/brdm}$, Cry2$^{-/-}$	24.4 ± 0.6	25.6 ± 3.1. then arrhythmic
Per1$^{brdm/brdm}$, *Per2*$^{brdm/brdm}$	Arrhythmic	Arrhythmic

SOURCE: Brown *et al.*[98]

mice.[17] Similarly, the tau mutation in Syrian hamsters that results in shortening of the free-running period of behavior also affects the SCN and peripheral tissues differently.[102]

Thus, the correlation between the circadian period of behavior or SCN electrical firing and the circadian period of peripheral gene expression is not exact. Indeed, neither period is an exact value. Different free-running periods of behavior can be measured for human beings kept under conditions of forced desynchrony (a day/night cycle so long or short that the endogenous circadian clock "free-runs" to reflect its endogenous period rather than adjusting to the environment) and under constant conditions.[103,104] Similarly, the period of circadian gene expression in fibroblasts can be altered by changing growth conditions such as incubation temperature and media supplements like serum (A. Dumas and S.A. Brown, unpublished observations).

Importantly, however, these properties appear to be traitlike; under similar conditions, they remain constant for a given individual. Our laboratory has been able to measure the period length of circadian gene expression in fibroblasts by using lentiviruses containing a circadian reporter (FIG. 3). Populations of cells from different individuals measured in this fashion showed an average period that corresponded to what has been measured for human behavior in other studies, but a standard deviation that was much broader among different individuals, implying the same sort of peripheral cell "exaggerations" to which we have alluded earlier.[98] We feel that this enhancement of individual differences makes the fibroblast period a good choice for a quantitative trait in genetic mapping studies to find the genes responsible for differences in human circadian behavior.

Such differences are likely to arise from a variety of different underlying genetic causes. A recent study of fibroblast circadian clocks in human morning-types and evening-types showed not only period differences among cell cultures from some individuals in the two groups, but also differences in the amplitude of circadian gene expression and in the phase-response properties of cells from people with opposite behaviors but identical free-running fibroblast periods.[105] Specifically, it was possible to determine that PRCs subject fibroblasts to forskolin, a chemical stimulus that activates adenyl cyclase by a mechanism reminiscent of the actions of the photopigment melanopsin upon the circadian oscillator. These curves clearly show that the phase of circadian gene expression can be altered by factors other than endogenous period length. Indeed, it has been shown before that the reduction of circadian amplitude in mice containing a mutant CLOCK allele can enhance phase shifting by light in these mice.[106] Our results using human peripheral fibroblasts imply that human circadian behavior may be determined by a rich mixture of causes, including the period length, amplitude, and phase-resettting properties of the endogenous circadian oscillator, and that these properties can be studied in peripheral fibroblasts.

It is possible that fibroblasts or other peripheral cell types might be used not only in the mapping of the genetic variations responsible for differences in human daily behavior, but also for the diagnosis of underlying causes of human circadian disorders in some individuals. For example, Vanselow and colleagues introduced a mutation in *Per2*, believed to be responsible for human FASPS, into fibroblasts, and were able to recapitulate the phase advance in the behavior of FASPS patients as an advanced phase of clock-gene transcription in synchronized FASPS fibroblasts. Subsequent molecular analyses allowed them to show effects of this mutation upon phosphorylation at multiple sites in the PER2 protein, and to further demonstrate that these modifications affected both PER2 protein stability and nuclear localization.[107]

Peripheral Oscillators as Biochemical Tools

Because the central clock in the SCN consists of only a few thousand neurons, it is a difficult subject for biochemical studies. By contrast, cells containing peripheral oscillators are readily available in large quantities from a variety of tissues. Thus, they make ideal subjects for biochemical investigations. Such oscillators are even present in immortalized cell lines,[108] thereby further permitting the easy introduction of genetic material into cells by transfection or transduction. Our laboratory has used this approach to purify proteins associated with PER1 from cultured 3T3 immortalized mouse fibroblasts. After stably transfecting the cells with an epitope-tagged *Per1* transgene, successive affinity and gel-filtration steps were used to isolate a PER1 complex whose components were subsequently identified by mass spectrometry. In this fashion, NONO and WDR5 were found as novel PER1-associated factors.[109] Previous studies of NONO (also known as $p54^{nrb}$ in humans) showed that it can affect various aspects of RNA metabolism[110] and nuclear retention.[111] WDR5, by contrast, was previously identified as a component of a Histone H3-K4 methyltransferase complex.[112]

To test the function of these newly identified proteins in the circadian oscillator, 3T3 cells were again

probed, this time via RNA interference and chromatin immunoprecipitation techniques. Such tools provide powerful methods to analyze the importance of both *cis*-acting elements and *trans*-acting factors to the circadian clock. For example, Ripperger *et al*.[26] identified the functions of various E-box elements within the circadian gene *DBP* by introducing marked and modified copies of the gene into fibroblast cells and studying their function. Similarly, Cavadini *et al*. showed that TNFα is able to suppress the expression of clock-genes in fibroblasts by inhibiting E-box-mediated transcription.[113] Fibroblast-based studies also demonstrate PGC-1α, a transcriptional coactivator important for energy metabolism, to be necessary for cell-autonomous clock function,[114] and highlighted the importance of the novel protein CIPC to circadian function.[20]

Conclusions

Circadian clocks have pervasive effects upon human physiology and behavior. Nevertheless, the complex and hierarchical nature of the mammalian circadian oscillator has long proven a barrier to its understanding at a molecular level. The discovery a decade ago of "slave" oscillators in most of the cells of the body has proven a turning point in our understanding. Peripheral clocks, and their communication with the SCN master clock, are essential to the regulation of circadian physiology in mammals. Equally important, they have proven a useful model system to probe the molecular roles of novel proteins within the oscillator. Finally, the similarity between the timing of peripheral circadian gene expression and the timing of daily human behavior may even render them useful in the quest for the genetic origins of human chronotype.

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Competing Interest

The authors declare no competing interest.

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